

**ANALYSIS OF THE BACTERIAL
COMMUNITIES ON PAINTINGS AND
ENGRAVINGS IN DOÑA TRINIDAD CAVE
(ARDALES, MALAGA, SPAIN)**

F. Stomeo, J.M.
Gonzalez, C. Saiz-
Jimenez.



*Instituto de Recursos Naturales y
Agrobiología, CSIC, Sevilla, Spain.*

Introduction

Caves often contain rock art paintings some of them dated from the Palaeolithic period, over 20,000 years old. At present, most known rock art paintings are affected by progressive biodeterioration (Holden 2002, 2003, Allemand and Bahn 2005, Sire 2006) and the control and preservation of these environments is a serious problem gathering the interest of scientists and conservators. In the Mediterranean area, several examples of Palaeolithic paintings have been found in caves such as La Pileta and Doña Trinidad, both in Malaga (Spain).

Microorganisms often colonize cave walls. This colonization can result in serious negative effects on the conservation of these prehistoric sites. As a result, the type and number of microorganisms should be studied in order to attempt their control and design preservation strategies (Gonzalez and Saiz-Jimenez 2004). A number of techniques are being developed to study the microbial communities in natural environments (Gonzalez and Saiz-Jimenez 2004). The use of culturing methods results in the detection of a low number of microbes from these environments. Recently, molecular techniques, based on the information present in nucleic acids, are gaining importance since they allow the detection of microorganisms without the requirement for growing them on culture media (Ward et al. 1990). In the last years, scientists are introducing the possibility of detecting the microorganisms present in an environment through molecular analyses based on DNA, as well as the microorganisms showing metabolic activity at the same site by RNA-based molecular studies. While the DNA is present in every organism at a relatively constant rate, the abundance of RNA per cell

is proportional to the metabolic activity and growth developed by that organism (Molin and Givskov 1999, Mills et al. 2004). Using molecular methods we can assess those microorganisms present in an environment as well as those showing activity and actually participating in the colonization processes going on at the studied site.

This study represents a first attempt to understand the microbial communities present in the Cave of Doña Trinidad, Ardales, Spain.

This cave was discovered in 1821, but it was not until 1918 that H. Breuil found and studied its prehistoric paintings and carvings. The cave has a length of 1.5 Km and contains several chambers and galleries. The interior contains labyrinths of columns, an underground lake, and beautiful formations of stalactites and stalagmites. The cave contains paintings and engravings which date back about 20,000 years old belonging to the Upper Paleolithic Age (Cantalejo et al. 2006). Herein, we focus on the detection of specific microbial groups and the analysis of their diversity in order to evaluate their potential risks involved in the conservation of the cave.

Materials and methods**Sampling**

Two sampling sites were selected for this study: L-12, a red-pigmented area in the cave wall, and M-13, a dust deposit collected from the engravings of snakes. Samples were collected under aseptic conditions into sterile tubes. Samples for culturing procedures were stored on ice while those for nucleic acid analyses were preserved in RNA lysis solution (Ambion, Austin, TX, USA) to avoid degradation by RNases and maintained at -80°C.

Culturing methods

The medium used was TSA (Trypticase-Soy Agar) which contains a complex composition based on peptone-like compounds suitable for the growth of a wide variety of heterotrophic microorganisms growing under aerobic conditions. Incubation of these cultures was performed at 28°C for up to one week. Differential colonies detected on the solid media were transferred to fresh media, isolated and identified by 16S rRNA gene amplification and sequencing as described below.

Extraction of nucleic acids

DNA was extracted using the NucleoSpin Food DNA extraction kit (Macherey-Nagel, Duren, Germany) following the manufacturer's recommendations. Total RNA was extracted using the RNeasy4PCR kit (Ambion, Austin, TX, USA). The protocol for total RNA extraction includes DNaseI treatment to remove any DNA present in the final RNA extract. A reverse transcriptase reaction was carried out to obtain the complementary DNA (cDNA) to the 16S rRNA genes to be amplified. The reverse transcriptase Thermoscript (Invitrogen, Carlsbad, CA, USA) was used in this study with a 16S rRNA gene-specific primer, 518R (5'-ATT ACC GCG GCT GCT GG), at an annealing temperature of 55°C for 1 h. A standard amplification reaction by PCR followed this step.

DNA amplification

Amplification of bacterial 16S rRNA fragments from genomic DNA was performed by PCR using the primer pair 27F (5'-AGA GTT TGA TYM TGG CTC AG) and 907R (5'-CCC CGT CAA TTC ATT TGA GTT T) from extracted DNA and 27F and 518R (Gonzalez et al. 2003) from cDNA. ExTaq (Takara, Shiga, Japan) was the DNA polymerase used for PCR, following the manufacturer's recommendations. Thermal conditions for the amplification reaction consisted on the following steps: 95°C for 2 min; 30 cycles (unless otherwise stated) of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min; and 72°C for 10 min.

Molecular fingerprinting

Fingerprints of the microbial communities were obtained by Denaturing Gradient Gel Electrophoresis (DGGE) following the method described by Muyzer et al. (1993) and Gonzalez and Saiz-Jimenez (2004). The primer pair used for amplification of DGGE suitable DNA fragments was: 341F-GC (5'-CC TAC GGG AGG CAG CAG and a GC-rich tail attached at its 5' end) and 518R (Muyzer et al. 1993, Gonzalez and Saiz-Jimenez 2004). Amplification conditions were as described above with the exception of an extension step of only 30 seconds. Migration markers (*Escherichia coli* and a *Streptomyces* sp.) were used throughout this study as reference for locating the position of cloned fragments in a microbial community fingerprint obtained by DGGE and facilitate sample comparisons.

Cloning and identification of microorganisms

PCR products were purified by the JetQuick PCR purification kit (Genomed, Bad Oeynhausen, Germany) and cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). The 16S rRNA libraries obtained were used to identify the microbial components of the community. A previously described screening method (Gonzalez et al. 2003) was followed with these libraries. Selected clones were sequenced.

Nucleotide sequence analysis

Sequence data was edited using the software Chromas, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm (Altschul et al. 1990) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/Blast/>). Sequences were checked for putative chimeras using Ccode as described by Gonzalez et al. (2005).

Results and discussions

Both culturing and molecular methods were applied to the study of the microbial communities in samples from the Cave of Doña Trinidad. Culturing methods allow the detection of the fraction of microorganisms present in the sample and able to grow on the medium and conditions provided in the laboratory. Among the microorganisms detected by culturing methods the most abundant were the Actinobacteria and Firmicutes. The most frequently encountered genera in samples L-12 and M-13 were *Streptomyces* (the most abundant), *Micromonospora*, and *Saccharothrix*, all three belonging to the Actinobacteria, and the genera *Bacillus* and *Paenibacillus* among the Firmicutes. Other cultured microorganisms often found in the cave were the Gamma-Proteobacteria (e.g. *Pseudomonas*) and Beta-Proteobacteria (e.g. *Ralstonia*). Red-pigmented areas of the cave walls were also represented by cultured microorganisms belonging to the Alpha-Proteobacteria (genera *Ochrobactrum* and *Rhizobium*). In sand and dust deposits collected from the engraving cavities we detected by culturing methods a significant proportion of Gamma-Proteobacteria of the genera *Pseudomonas* and *Acinetobacter*. Figure 1 shows microbial community fingerprints of the two studied samples and the identification of some of the detected

DNA bands from cultures and a DNA-based molecular survey.

Molecular techniques revealed the presence of a relatively diverse microbial community. The detected microorganisms belong to the groups Alpha-, Beta-, and Gamma-Proteobacteria, the Firmicutes, Actinobacteria, and Bacteroidetes. The most abundant were the Alpha-Proteobacteria representing around 46% of sequenced clones, followed by the Gamma-Proteobacteria with a 27% of sequences. Other groups showing around 8% of sequenced clones were the Firmicutes and the Beta-Proteobacteria. The Bacteroidetes and Actinobacteria were also significant, representing over 5% of sequences.

DNA- compared to RNA-based analysis showed the presence of metabolically active Alpha-Proteobacteria in a high proportion (over 50% of sequenced clones) in a sample taken from deposits on the snake-shaped engravings. Other bacterial group detected from a RNA-based molecular survey were the Gamma-Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Thus, Alpha-Proteobacteria might be playing a critical role in Doña Trinidad Cave biogeochemistry.

Comparisons of culturing vs. molecular methods (both DNA- and RNA-based strategies) revealed clear differences among the bacterial groups detected. For instance, several bacterial phyla had never been isolated before even if they are frequently detected by the culture-independent methods used during this study. Most bacteria detected by molecular methods were related to previously uncultured microorganisms. Therefore, defining the potential role of these microorganisms in cave biodeterioration and biogeochemistry is not an easy task. Further research and efforts on the culturing of these microorganisms is required.

Cave microbiology is strictly regulated by environmental factors. While most caves, including Doña Trinidad Cave, show fairly constant conditions throughout the year, changes in that equilibrium could result in fluctuations in the microbial community composition. Strict management should be reinforced to maintain the natural conditions of this cave in order to avoid accelerated and unpredicted negative microbial effects on its conservation.

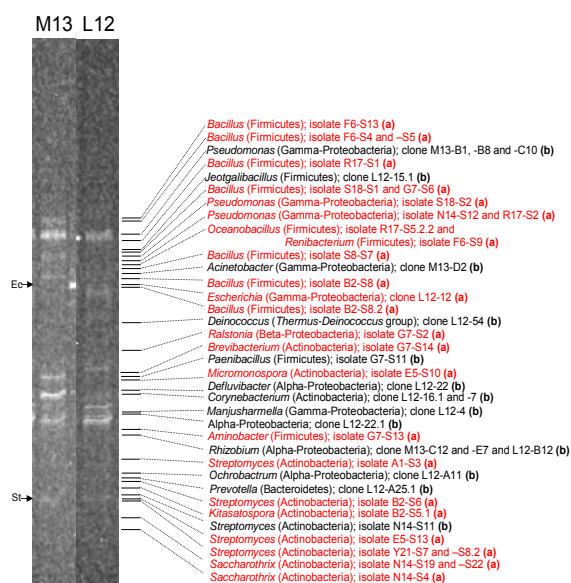


Figure 1. Microbial community fingerprints by DGGE analysis of two samples from Doña Trinidad Cave and the location of the DNA band (16S rRNA gene fragment) corresponding to the identified microorganisms. These microorganisms were either cultured (a) or detected from DNA-based molecular surveys (b). M-13 sample corresponded to material collected from the engraving cavities; L-12 corresponded to a red-pigmented area. Arrows indicate the location of two migration markers corresponding to *Escherichia coli* (Ec) and *Streptomyces* sp. (St).

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References

- Allemand, L., Bahn, P.G. (2005). Best way to protect rock art is to leave it alone. *Nature* 433: 800.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.
- Cantalejo, P., Maura, R., Espejo, M.M., Ramos, J.F., Medianero, J., Aranda, A., Durán, J.J. (2006). *La Cueva de Ardales: Arte prehistórico y ocupación en el Paleolítico Superior. Estudios, 1985-2005*. Málaga: Diputación Provincial.
- Gonzalez, J.M., Saiz-Jimenez, C. (2004). Microbial diversity in biodeteriorated monuments as studied by Denaturing Gradient Gel Electrophoresis. *Journal of Separation Science* 27: 174-180.
- Gonzalez, J.M., Ortiz-Martinez, A., Gonzalez-delValle, M.A., Laiz, L., Saiz-Jimenez, C. (2003). An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. *Journal of Microbiological Methods* 55: 459-463.
- Gonzalez, J.M., Zimmermann, J., Saiz-Jimenez, C. (2005). Evaluating putative chimeric sequences from PCR amplified products and other cross-over events. *Bioinformatics* 21: 333-337.
- Holden, C. (2002). Cave paintings in jeopardy. *Science* 297: 47.
- Holden, C. (2003). Wanted: solution for cave mold. *Science* 300: 245.
- Mills, H.J., Martinez, R.J., Story, S., Sobczyk, P.A. (2004). Identification of members of the metabolically active microbial populations associated with *Beggiatoa* species mat communities from Gulf of Mexico cold-seep sediments. *Applied and Environmental Microbiology* 70: 5447-5458.
- Molin, S., Givskov, M. (1999). Application of molecular tools for in situ monitoring of bacterial growth activity. *Environmental Microbiology* 1: 383-391.
- Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59: 695-700.



Sire, M.A. (2006). Des restaurateurs au chevet des peintures de Lascaux. De l'élimination des champignons au constat d'état. *Monumental* 2006 Semestriel 2 : 68-75.
Ward, D.M., Weller, R., Bateson, M.M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345: 63-65.

CHARACTERIZATION OF MODIFICATIONS IN POLYMER FILMS BY LASER INDUCED FLUORESCENCE

M. Walczak, M. Oujja,
S. Gaspard, E. Rebollar,
M. Castillejo



*Instituto de Física Química Rocasolano, CSIC,
Madrid, Spain*

Introduction

Laser Induced Fluorescence (LIF) technique, due to its sensibility and non destructivity, is an important tool used within variety of applications, such as biochemistry, biophysics and medicine. LIF can be used to investigate the chemical and physical properties of organic compounds; however in the case of polymers, the intrinsic fluorescence is unspecific, so in order to analyze their properties by LIF the incorporation of fluorescent additives is necessary.

In this work we report on two applications of this powerful technique. We used LIF to study the photochemical modifications induced in doped polymer films by irradiation with CO₂ laser and to characterize the fluorescent response of polymeric sensors to the presence of organic contamination.

Study of photochemical modifications induced in polymer films by irradiation with CO₂ laser

Laser polymer ablation and processing offers a broad spectrum of scientific applications covering removal, surface modification and film deposition [Lippert et al. 2003]. The objective of the work presented in this paragraph was to investigate the mechanisms of interaction between IR laser irradiation and polymer films. We study the TEA CO₂ laser ablation of films of poly(methyl methacrylate), PMMA, with average molecular weight 996 kDa (Aldrich) doped with photosensitive compounds iodo-phenanthrene (PhenI) and iodo-naphthalene (NapI) from Aldrich. Such

system can be considered as a simple model of an artistic paint layer where pigment particles are immersed in a polymer matrix.

Films were prepared by casting on CaF₂ substrates solutions of the purified polymer and of the dopant dissolved in dichloromethane to a concentration of 1.2 wt.%. Typical film thicknesses were in the range of 90 to 120 µm.

The targets were irradiated in air by the focused output of a CO₂ laser (Lumonics K-103 TEA, 100 ns fwhm) tuned to a wavelength of 10.59 µm in resonance with the γ(CH) mode of the dopants and with the O-CH₃ rock mode of PMMA [Gaspard et al. 2007]. The linear absorption coefficients of the prepared films at the irradiation wavelength were around 150 cm⁻¹ (as measured on a Perkin Elmer 1600 IR spectrometer).

The formation of photoproducts in irradiated areas of the films was monitored by LIF. A pump-probe scheme is employed in which the CO₂ laser pumps the samples and fluorescence of photoproducts is induced by excitation at 266 nm with the frequency doubled output of a dye laser. Both laser beams are incident at 45° onto the sample (the pump beam focused down to ≈ 5 mm² and the probe beam to a somewhat smaller size). A delay unit (SR DG535) controls the pump and probe time delay and the gating of the detection system. The induced emission from the area of the sample irradiated by a single pulse is collected, perpendicularly to the propagation direction of the lasers, by a f = 4 cm focal length lens onto the entrance slit of a 0.30 m grating spectrograph (TMC300 Bentham, 300 grooves/mm) and the spectrum is recorded by a time gated ICCD detector (2151 Andor Technologies) interfaced to a computer. A cut-off filter (>300 nm) is used for blocking any probe beam scattered light. ICCD delay and detection gate of 0 and 100 ns respectively are employed for fluorescence detection (corresponding to the lifetime of ArH [Michaelian et al. 1973]).

Monitoring of the LIF spectra reveals the formation of photoproducts on the irradiated areas of the films. Similarly to what is observed upon UV irradiation (Rebollar et al. 2006a) the spectra of PhenI and NapI doped